STUDIES OF NITROGEN FIXATION

I. NITROGEN FIXATION IN THE DEAD LEAVES OF FOREST BEDS

BY

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INTRODUCTION

It is known that processes are continually taking place in nature whereby small amounts of free atmospheric nitrogen are converted to a combined state. This takes place partly by electric discharges in the atmosphere, giving rise to the formation of small quantities of nitric and nitrous acids, and partly by biological processes, since certain bacteria (and possibly also fungi and blue-green Algae see Drewes 1928, Allison and Morris 1930) can employ free atmospheric nitrogen in building up their proteins. These nitrogen-fixing bacteria live partly free in the soil, and partly in the roots of certain species of plants. Thus it is well known that the roots of leguminous plants are furnished with nodules containing bacteria belonging to the group Bacterium radicicola. The best known free living nitrogen-fixing bacteria are species of the genus Clostridium and Azotobacter. The Clostridium forms are distributed in all kinds of soil, while the Azotobacter forms are only found in soil having a p_H of 6 or higher.

It is known that in the case of cultivated land quite considerable amounts of free nitrogen (100-200 kg per hectare) can be converted to a combined state by a crop of leguminous plants. On the other hand in spite of numerous investigations of the physiology of the free living nitrogen-fixing bacteria, not much is known about their effectiveness in fixing nitrogen in the soil, and it is this question which will be dealt with in the following sections.

A. General considerations on the action of free living nitrogen-fixing bacteria in nature.

a. *Which organic substances serve as a source of carbon for free living nitrogen-fixing bacteria under natural conditions?*

In artificial cultures containing mannite or monosaccharides as a source of carbon, but not containing combined nitrogen, it is known that Clostridium and Azotobacter are able to utilize free atmospheric nitrogen in building up their proteins. In nature, however, the bacteria must use other sources of carbon than mannite and monosaccharides, since these substances are practically speaking non-occurrent in soil. Little is known, however, about which substances can be considered as possible sources of carbon under natural conditions. They may be partly organic substances directly given off by the roots of the living plants, and partly substances formed by decomposition of the dead plants.

A few investigations have been carried out recently which seem to indicate that the roots of the higher plants can give off organic substances which can be utilized by free living nitrogen-fixing bacteria. Truffaut and Bezssonoff (1925, 1926, 1927) have shown that it is possible to grow maize plants and bring them to full normal development in sterile sand cultures containing only the necessary mineral nutrients but no combined nitrogen, if the otherwise sterile cultures are inoculated with one or more species of free living nitrogen-fixing bacteria. Under these conditions the bacteria have thus been able to develop and flourish and also supply the growing maize plants with nitrogen compounds, although no organic substances were added to the culture. The bacteria must thus have obtained the organic substances necessary for nitrogen fixation from the maize plants, which must have given them out through the roots. It is not possible to give plants nitrogen nutrition in this way in nonsterile sand-cultures, probably because under such conditions other bacteria consume the substances which should serve as source of carbon for the nitrogen-fixing bacteria.

A somewhat similar result has been obtained by Caron (1923, 1930) who has succeeded in isolating from compost and stable manure a species of bacteria which when added to sterile cultures of barley grown in gravel seems to be able to convert atmospheric nitrogen to a form in which the barley plants are able to assimilate it. The bacteria in question is commonly found in stable manure and in meadow and arable land, although in very varying amounts, and Caron found it difficult to avoid infection by this bacteria in control experiments. In the series in which the control experiments were quite sterile the plant growth in the inoculated cultures was three times as great as that in those not inoculated. The question of to what extent there is an actual symbiosis between the barley plants and the bacteria has not been investigated, nor whether the bacteria are able to fix atmospheric nitrogen when in pure culture.

It remains however an open question to what extent free living nitrogen-fixing bacteria use organic substances given off by plant roots in the soil under natural conditions. Several workers have shown (Schloesing and Laurent 1891, 1892, Wilfarth and Wimmer 1907) that nitrogen fixation can take place in moist soil poor in nitrogen which is overgrown with green algae. This nitrogen fixation is due to free living nitrogen-fixing bacteria, and the necessary organic substances are provided by the algae. It is not however known whether it is the living algae cells which provide the bacteria with organic matter, or whether the bacteria live on substances originating in the dead algae cells.

As previously mentioned, substances formed by the decomposition of dead plants can also be considered as possible sources of carbon for the free living nitrogen-fixing bacteria. It has previously been thought that the actual humus matter (the dark coloured organic constituents of the soil) could serve as sources of carbon for Clostridium and Azotobacter (Heinze 1904, Hanzawa 1914), but Krzemieniewski (1909) has shown that this is not the case. While the addition of solid or dissolved humus to artificial cultures of Azotobacter containing mannite certainly does cause a considerably increased nitrogen fixation, no fixation takes place when the mannite is omitted, even in the presence of large quantities of humus. Krzemieniewski (1909) and Remy and Rösing (1911, 1912) have shown that it is the iron in the humus which causes the increased nitrogen fixation in the cultures containing mannite, and that iron-free humus is without action. Humus matter as such can thus not be utilized by Azotobacter as a source of carbon. It is therefore impossible as a rule to detect any increase in combined nitrogen on storing soil containing humus for long periods. The numerous investigations in the literature (see e. g. Bradley and Fuller 1930) on the so called nitrogen-fixing power of different types of soil have not been carried out in this way, but by transferring a portion of the soil to a culture containing mannite, and determining the nitrogen content at once and after a certain period. The increase in nitrogen is then calculated per kg of dry soil, on the basis of the amount added to the mannite solution. Such investigations are obviously not of great interest, since they can only show that the soil contains bacteria which are able to fix atmospheric nitrogen when transferred to a mannite solution, but can give no information as to how far such fixation takes place in the soil under natural conditions.

While the free, nitrogen-fixing bacteria are thus unable to utilize the actual humus matter as a source of carbon, it seems that substances formed by the decomposition of certain dead vegetable substances such as straw, withered leaves, etc., can be used in this way. Experiments on nitrogen fixation with the addition of such natural products have been carried out by Hutchinson (1918) who mixed 5—10 % of hay or straw with the samples of soil and obtained increases of 8—40 % in the content of combined nitrogen in the mixtures. The greatest increase in nitrogen was found in a mixture of 5 g earth, 5 g sand, 1 g straw and 1 g calcium carbonate. This mixture originally contained 15.3 mg nitrogen, and after 69 days 21.57 mg. — an increase of 6.27 mg N. It is probably the decomposition products of the cellulose from the straw which are utilized by the nitrogen-fixing bacteria, since both Clostridium americanum (Pringsheim 1909) and Azotobacter (Tuorila 1928) can fix nitrogen in artificial cultures, but only when the cultures also contain bacteria which decompose cellulose, since the nitrogen-fixing bacteria themselves are not able to attack the cellulose. Koch (1910) has shown later that a comparatively large nitrogen fixation can take place in soil to which paper has been added, when besides nitrogen-fixing bacteria, cellulose-decomposing bacteria from horse manure are added.

Investigations of nitrogen fixation with natural vegetable material (fallen leaves from forest trees) have also been carried out by Henry and by Hornberger, whose experiments will be discussed later.

b. The behaviour of free living nitrogen-fixing bacteria with regard to fixed nitrogen.

A point which is often overlooked, but which must be taken into consideration in all investigations of nitrogen-fixation and in assessing the activity of the free living nitrogen-fixing bacteria in nature, is that these bacteria can also use ammonium salts and nitrates as a source of nitrogen (as shown by Beijerinck and Van Delden as early as 1902) and only have recourse to free atmospheric nitrogen when these compounds are absent or present in insufficient amount.

According to experiments carried out by Burke and Lineweaver (1930) Azotobacter in artificial cultures ceases to fix atmospheric nitrogen when there is present more than 0.5 mg nitrogen per 100 cc solution as ammonia or nitrate[1]).

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[1]) Kostytschew, Ryskaltschuk and Schwezowa (1926) give 15 mg nitrate or ammonia-nitrogen as the critical value at which nitrogen fixation by Azotobacter ceases, and the corresponding value for Clostridium americanum is given as 4 mg. nitrate-nitrogen per 100 cc solution by Pringsheim

In forest soil and in well manured garden and meadow soil considerable amounts of nitrate are formed by nitrification, and the nitrate content of the soil is doubtless never so low that the nitrogen-fixing bacteria need have recourse to free atmospheric nitrogen. On account of this fact some writers have doubted or denied that Azotobacter and Clostridium fix elementary nitrogen when they live under natural conditions in the soil. Thus Thiele (1906) considers the power of fixing nitrogen by Azotobacter as a latent power which only functions when the bacteria is given dissolved carbohydrates or mannite in the laboratory, and simultaneously starved with regard to nitrogen, which conditions it never meets with in the soil. Similar views are held by Bonazzi (1921).

Since the free living nitrogen-fixing bacteria thus prefer ammonium salts and nitrates to elementary nitrogen as a source of nitrogen, it is easily understood why an increase in the amount of combined nitrogen is hardly ever observed on storing samples of soil. Such storing almost always gives rise to accumulation of nitrates (or ammonia in the case of strongly acid forest soil) and the presence of these compounds will prevent fixation of free nitrogen. Nitrogen fixation can then only take place when a large amount of organic material containing little or no nitrogen (straw, cellulose, etc.) has been added to the soil, as in the experiments of Hutchinson previously mentioned. The reason for this is that during the decomposition of the organic substance poor in nitrogen not only are substances formed which serve as a source of carbon for the nitrogen-fixing bacteria, but there is a simultaneous disappearance of all the ammonia- and nitrate-nitrogen present in the soil, caused by its consumption by the organisms which break down the nitrogen-free substance (especially cellulose). The ammonia- and nitrate nitrogen is transformed to protein nitrogen in the bodies of these organisms. The micro-organisms

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(1914). The fact that these workers have obtained values considerably higher than those of Burk and Lineweaver is due to the fact that they have worked with cultures which have stood a long time. Under such conditions the bacteria will soon have consumed all the combined nitrogen in cultures where the initial concentration is low, after which nitrogen fixation can take place. Burk and Lineweaver, on the other hand, have made experiments of short duration, in which the amount of nitrogen fixed could be followed from hour to hour. This was done by means of a special gas-volumetric micro-apparatus.

which decompose cellulose consume one part of nitrogen for every 30 parts of cellulose broken down (Waksman 1928).

As long as the decomposition of the material containing cellulose lasts, the soil will remain entirely free from ammonium salts and nitrates, and this may take up to a year, varying with the temperature, and the nature of the material added (Söderbaum and Barthel 1924, Viljoen and Fred 1924, Anderson and Arlington 1925). Only when the decomposition of the cellulose is complete can ammonia- or nitrate nitrogen again be detected in the soil.

There is in fact in the soil, as in all natural processes where organic matter is decomposed, a fairly constant ratio between the amounts of carbon and nitrogen in the organic matter, and this ratio is about 10:1 (see e. g., Waksman 1924, 1927, Leighty and Shorey 1930). If the ratio between the amounts of carbon and nitrogen is greater than 10:1, i. e., the organic matter is relatively poor in nitrogen, the nitrogen is kept, since the nitrogen compounds liberated during the decomposition processes are continuously taken up by the micro-organisms, while the amount of carbon is decreased by loss of carbon dioxide. When the amount of carbon has decreased in this way so much that the ratio between carbon and nitrogen in the organic matter has fallen to 10:1 or less, only a part of the nitrogen in the nitrogeneous compounds formed in the decomposition is used by the micro-organisms, the remainder being converted to ammonia nitrogen, which is then oxidised to nitrate by nitrifying bacteria. The nitrate nitrogen is then either removed by higher plant forms, or is carried into lower layers of soil by the rain soaking through. Nitrogen is thus removed during the process of decomposition, and the ratio between carbon and nitrogen in the organic matter remains approximately constant at about 10:1.

Plant matter (e. g. straw) which contains less than 1.7 % nitrogen in the dry material does not contain enough nitrogen for a complete decomposition at a reasonable speed (Waksman and Tenney 1928). In the absence of added combined nitrogen such material will therefore decompose slowly, and remain free from the least trace of ammonia or nitrate nitrogen. It is in just such material that the possibility exists of fixation of free atmospheric nitrogen by free living nitrogen-fixing bacteria

c. *Where does nitrogen fixation take place in nature?*

On the basis of the considerations developed above we shall now consider the problem of where we can expect to find in nature the necessary conditions for nitrogen fixation. If we exclude the nitrogen-fixation brought about in the root nodules of leguminous plants by the action of Bacterium radicicola, and the possibility that the same process may take place in the "Rhizosphere"[1] of the roots, the only localities which come in question are those where vegetable matter comparatively poor in nitrogen is accumulated in large amounts. Considerable amounts of nitrogen fixation will hardly take place in the soil itself unless it contains dead roots or is mixed with large quantities of vegetable matter (by the agency of human beings or rooting animals) and even under such conditions the nitrogen fixation is presumably localized in the neighbourhood of the dead vegetable matter. It is thus possible that in arable land the nitrogen fixation takes place in the remains of plants left after removal of the cereal crops, i. e. roots and stubble, which constitute a material comparatively poor in nitrogen. It has in fact been shown by **Hutchinson** in the experiments already described, that a considerable increase in combined nitrogen does take place in a mixture of soil and straw[2]. In meadows and other places covered continually with vegetation, as well as in the forest bed, a layer of withered plant remains (straw, leaves, etc.) is deposited each autumn, und there is thus a possibility for nitrogen fixation during the decomposition of this material the following spring and summer.

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[1] The term "rhizosphere" has been used by **Hiltner** (1904) to denote that part of the soil which is immediately in contact with the roots of the living plants. **Hiltner** assumes that the chemical, physical and micro-biological conditions in the rhizosphere are affected by the living plant roots and thus differ essentially from the conditions in the remainder of the soil. It has thus been shown that the number of bacteria (including the number of nitrogen-fixing bacteria, e. g. Azotobacter) is considerably greater in the immediate neighbourhood of the roots than at a distance. In order that nitrogen fixation should be possible in the rhizosphere, using organic substances given off by the roots, the conditions must of course be such that the nitrogen-fixing bacteria cannot obtain nitrate or ammonia nitrogen. Nothing is however known about this point, and the whole question needs further investigation.

[2] Nitrogen fixation can take place in fresh horse manure under certain conditions (**Richards** 1916), presumably when it is relatively poor in nitrogen, since the phenomenon is only observed in manure from horses which had a special diet.

d. The investigations of Henry and Hornberger on nitrogen fixation in the dead leaves of forest beds.

We shall in the following deal with investigations of how far nitrogen fixation takes place during the decomposition of the layer of leaves in forest beds. This question has previously been treated by Henry and Hornberger.

In Henry's first paper the following experiments are described:

The fallen leaves of oak and hornbeam trees were placed in metal boxes, which had a perforated bottom covered with chalk or sandstone. The boxes were covered with wire netting on top, and were kept in the open air at a height of 60 cm from the ground. The nitrogen content of the oak leaves was originally 1.108 °/o (calculated on dry material), and 1.923 °/o after one year. Simultaneously, however, a loss of 21.6 °/o of total dry matter had taken place. If, however, the amount of nitrogen found after one year is referred to the original amount of dry material, an absolute increase in nitrogen of 400 mg per 100 g dry material originally present is found, (or the amount of nitrogen originally present has increased by 36 °/o). In the case of hornbeam leaves, the nitrogen content increased from 0.947 °/o to 2.246 °/o in the course of a year, while the loss of dry material was 23 °/o. This gives an increase of 780 mg nitrogen per 100 g of dry material originally present (or the amount of nitrogen originally present has increased by 82 °/o). It is thus a question of extremely large increases in nitrogen.

In the paper published in 1904 Henry describes new experiments carried out in the same way with leaves of beech, hornbeam, oak, aspen, fir and pine. He now finds however considerably smaller increases in nitrogen than in the first experiments. Thus after standing for one year in the open air, the following results were obtained:

	N-increase per 100 g original dry matter	Original amount of N increased by
Beech	155 mg N	12.6 °/o
Hornbeam	0 - -	0.0 -
Oak	69 - -	6.0 -
Aspen (over chalk)......	343 - -	39.0 -

	N-increase per 100 g original dry matter	Original amount of N increased by
Aspen (over limestone) ..	84 mg N	9.6 %
Austrian fir	105 - -	23.7 -
Pine	125 - -	15.2 -

Hornberger writes however in 1905, that he has repeated Henry's experiments, using fallen leaves of oak, beech, pine, ash and acacia (robinia) as material. Although he followed exactly the directions given by Henry, he was not able to show that any nitrogen fixation took place during storage of the leaves, but found in fact that a loss of nitrogen took place in most cases. This loss constituted up to 9 % of the original amount of nitrogen[1]). In a later paper (1906) Hornberger succeeded in demonstrating an increase of nitrogen in isolated cases for fallen beech leaves (94 mg N per 100 g dry material, or 9 % increase on the original amount of nitrogen) and hornbeam leaves (109 mg N per 100 g dry material, or 8 % increase on the original nitrogen), but found a loss of nitrogen in just as many cases. Thus in one case hornbeam leaves lost 9 % of their original amount of nitrogen. Further, Ehrenberg (1907) in a review of Hornberger's paper writes as follows: »Abgesehen davon dass von den gesamten Versuchen des Verf. bisher nur 28 % einen Stickstoffgewinn, die übrigen aber Stickstoffverluste aufzuweisen hatten, sei darauf hingewiesen, dass die vom Verf. erreichte Genauigkeit bei der Analyse für so feine Unterschiede, wie sie hier in Frage kommen, kaum ausreichen dürfte«. This is illustrated by examples taken from the results of Hornberger's analyses. Thus it appears that in the experiment where an increase in nitrogen of 9 % is given, the two nitrogen estimations carried out at the conclusion of the experiment differ by 6 %, while the two corresponding estimations carried out with the initial material differ by 3.6 %. Double analyses were not carried out in determining the dry matter.

After the appearance of Hornberger's two papers, our confidence in the reliability of Henry's experimental results is naturally shaken, especially as he gives no details of how sampling and analysis was carried out. There are no subsequent investigations of the subject, and Henry's results thus remain still unconfirmed.

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[1]) The losses are greatest in the case of the leaves containing most nitrogen (ash and acacia leaves).

B. Investigations carried out in the Carlsberg Laboratory on nitrogen fixation in fallen leaves of the beech, oak and hornbeam.

a. Experimental method.

With the view of approximating as closely as possible to natural conditions, Henry and Hornberger placed their leaf matter in the open air in boxes raised 60 cm above the surface of the earth[1]. When the leaves are in open boxes only covered by wire netting there is always the possibility of contamination by dust, bird excrement, insects and other similar nitrogeneous material, and it is then naturally very problematic whether nitrogen fixation actually has taken place. Further, the above named authors did nothing to make their initial material as uniform as possible, which is of great importance for the nitrogen determinations. Preliminary experiments in the Carlsberg Laboratory with dried beech leaves showed that different 1 g samples taken from the same larger sample could show differences in nitrogen content of up to 14 %.

It will thus be realised that reliable results cannot be obtained by following the method given by Henry. It was therefore decided to give up the idea of experimenting under natural conditions in the open air, and in order to be able to demonstrate with certainty even small increases in nitrogen, it was found necessary to prepare a uniform mixture of the leaf material. It is in fact possible to weigh out a number of samples containing exactly the same amount of nitrogen when such a uniform material is used. When it is wished to investigate whether nitrogen fixation has taken place after storing for a suitable period, this can be done simply by taking out one of the samples and determining its nitrogen content. As the whole sample is used for the nitrogen determination, the latter is independent of a determination of dry matter, which is of course a great advantage. It is also of importance to follow during the course of the experiment the loss of dry matter, the percentage nitrogen in the dry matter, the p_H of the mixture and its content of nitrate or ammonia nitrogen, for which purposes special samples are used. A preliminary investigation had shown that on allowing fallen beech leaves, kept suitably moist with distilled water, to stand in jars in the laboratory, their p_H altered during the process of decomposition. The p_H value was originally 5.0, but rose in the course of a year to ca. 7.0. During this period the leaves remained absolutely free from ammonia and nitrate nitrogen, but later nitrification set in and the p_H value decreased on account of the accumulation of nitric acid in the leaves. The nitrogen percentage in the dry material increased from 1.2 to 2.0 during storage, but since the loss in dry material was not investigated, this experiment gives no information as to whether or not an increase of nitrogen has taken place.

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[1] It is also questionable whether the leaves can be considered »under natural conditions« when they are in boxes 60 cm above the earth. Among other things, they are certainly much more liable to be dried during dry periods than in the forest bed.

The material for the final experiments consisted of fallen leaves of beech, oak and hornbeam and was collected about Dec. 1 st 1930, the beech leaves in Ermelund, the oak leaves in a young oak plantation in Grib Skov, and the hornbeam leaves in the Fortunen enclosure. To each sort of leaves a small amount (ca. 0.1 %) of older material in vigorous decomposition was added. This added material, which was taken from the same places as the other leaves, should ensure the presence of the micro-organisms necessary for decomposition and nitrogen fixation, if any.

The leaves were cut up in their natural moist condition by an electric mincing machine, after which each portion was carefully mixed in order to obtain an absolutely uniform material. Portions were weighed out from these mixtures of 50 g (15.29 g. dry matter) in the cases of beech leaves and 40 g in the case of oak and hornbeam leaves (11.15 g. dry matter in the oak samples and 11.88 g in the hornbeam samples). For each sort of leaves four of these samples were taken to determine the initial nitrogen content, while the rest were kept under suitable conditions for later analysis with a view to detecting any increase in nitrogen.

The estimation of nitrogen. This was carried out according to a method given by Carsten Olsen (1927) which is a modification of Kjeldahls method, specially developed with a view to experiments on nitrogen fixation. The nitrogen found by this method includes any which may be present as nitrate. On account of the unusually large amount of organic matter to be decomposed, it was necessary to employ especially large Kjeldahl flasks, and correspondingly large quantities of reagents. The method of procedure was as follows:

The whole sample was transferred to a 500 cc Kjeldahl flask and there was added 20 cc conc. sulphuric acid and 60 cc water minus the amount of water previously found in the sample. 10 g. ferrum reductum (Merck, pro analysi) and some drops of octyl alcohol were then added. This causes a vigorous evolution of hydrogen, whereby any nitrate present is reduced to ammonia. When the hydrogen evolution began to subside the flask was warmed gently for 10 mins. and then 50 g potassium sulphate, 10 g cupric sulphate, 7.5 g mercuric sulphate and 110 cc conc. sulphuric acid added. The flask now stood over a small flame until the next day, in order to evaporate off the water present. The gas was then turned up and the decomposition carried out in the usual way until all the organic matter had disappeared. (About 30 cc conc. sulphuric acid was used up in oxidising the organic matter). The whole was then boiled for 3 hours. After cooling, the contents of the Kjeldahl flask were transferred to a litre measuring flask and filled up to the mark with water, care being of course taken that the temperature of the flask during the final addition of water was the same as that for which the flask was calibrated. Two 100 cc samples were taken from this mixture with a calibrated pipette, and used in the distillations. Blank experiments with the reagents alone were of course carried out parallel with the analyses. Reference may be made to the paper quoted above for further details[1]).

Estimations of the initial nitrogen content in the samples taken gave the following results:

50 g. beech leaves = 15.29 g. dry matter	40 g. oak leaves = 11.15 g. dry matter	40 g. hornbeam leaves = 11.88 g. dry matter
211.2 mg. N	135.1 mg. N	207.7 mg. N
211.3 - -	135.3 - -	208.0 - -
211.1 - -	135.0 - -	207.6 - -
211.4 - -	135.4 - -	207.7 - -

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[1]) Since nitrates were not present in the leaf material at the beginning of the experiments, a few experiments were carried out for the sake of comparison using the ordinary Kjeldahl method without the addition of ferrum reductum. but othervise using the same amounts of sulphuric acid and salt mixture as given above. The two methods gave results which did not differ more than the single estimations carried out by the same method.

These determinations show that the weighed out samples did not vary in nitrogen content more than 0.3 % among themselves. It should thus be possible to demonstrate with certainty a nitrogen increase of 1 %.

Estimation of dry matter. This was carried out by drying 20 g. leaf material in a vacuum drier at 80° for three days.

Estimation of hydrogen ion concentration. The p_H values were determined colorimetrically in an aqueous extract by the same method used for the estimation of p_H in soil (see Carsten Olsen and Linderstrøm-Lang 1927).

Tests for nitrate and ammonia. An aqueous extract of the leaf material was tested for nitrate with diphenylamine-sulphuric acid, and for ammonia with Nessler's reagent.

b. Investigations of nitrogen fixation in beech leaves.

60 50 g portions of the cut and well mixed beech leaves were weighed out 9/12 1930. As stated above, each portion contained 15.29 g dry matter and 211.3 mg nitrogen. Immediately after weighing, each portion was transferred to a 500 cc glass-jar covered with a glass plate. The jars were arranged in four series. In series 1 and 3 the material had a p_H of 4.8, while in series 2 and 4 3 g calcium carbonate[1]) was added to each portion, which altered the p_H value to 7.6. Distilled water was added to the series 1 and 2 in such quantity that the sample contained 75—80 % water, which corresponds approximately to the average water content of the decomposing leaves in the forest bed. It was attempted to maintain this content of water throughout the whole experiment by adding distilled water as soon as the material was judged to be too dry. The decomposition of the leaf material in series 1 and 2 can be said to have taken place under aërobic conditions. In series 3 and 4 so much water was added that all air was excluded and the consistency of the mixture was that of thin porridge (ca. 90 % water). The decomposition of the leaves in these two series of experiments thus took place under anaërobic conditions.

Besides the four series of experiments already mentioned, two other series (5 and 6) were carried out in which the leaf material was mixed with forest soil. Each portion in these two series contained 12 g leaves (3.67 g dry matter containing 50.7 mg nitrogen) and 25 g forest soil (21.65 g dry matter containing 45.45 mg nitrogen). The forest soil had a p_H value of 5.4 and was taken from the same place in Ermelund as the beech leaves. Stones and needles were removed by sifting, and the soil was then carefully mixed before weighing out the samples. The mixtures of soil and leaf had a p_H of 5.2 (series 5). 2 g calcium carbonate was added to each of the samples in series 6, which changed the p_H value to 7.6. The water content of the mixtures of soil and leaf in series 5 and 6 was ca. 44 %, and it was sought to maintain this content of water throughout the experiments.

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[1]) The calcium carbonate was distributed evenly throughout the sample by means of a spatula.

All the 6 series of experiments were commenced $^9/_{12}$ 1930. The jars were kept in a cupboard at a temperature of 22° C. The experiments were concluded $^{10}/_{11}$ 1931 after 337 days. During this period (as shown in Tables 1 and 2) one portion was taken from each series at intervals for the determination of nitrogen, and simultaneously samples were taken for the measurement of p_H[1]. Portions were also taken at three different times for the estimation of dry matter.

At the beginning of the experiments 0.5 g sublimate (dissolved in water) was added to one portion in each series in order to destroy all micro-organisms. These portions stood in the same cupboard as the remainder and were used in nitrogen estimations at the conclusion of the experiments. It is seen from Table 1 that the nitrogen content of these portions was the same as at the commencement of the experiments. This shows that the jars have not acquired nitrogen from the air in the form of ammonia or nitric acid, but that the increases of nitrogen found are due to the action of micro-organisms.

The experimental results are given in tables 1—5 and figures 1 and 2, from which it seems that nitrogen fixation has taken place in all six series of experiments. The nitrogen increase is greatest in series 4, where a fixation of 180 mg nitrogen per 100 g of the original dry matter has taken place, which

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[1] In the case of series 1, three portions were taken out each time, since this series consisted of three times as many portions as the remainder. It was originally intended to divide this series into three parts, to two of which various inorganic salts should be added, but this plan was relinquished.

Table 1.

The nitrogen content (expressed as mg N) in the weighed out portions of beech leaves at 6 different times during the duration of the experiment.

No. of days ⟶		0	36	95	161	229	337	Sublimate added
Series 1	50 g leaves (15.29 g dry matter), aërobic conditions	211.3	211.3 211.4 211.3 M: 211.3	211.0 211.1 211.3 M: 211.1	216.0 217.3 215.1 M: 216.1	218.7 219.5 218.8 M: 219.0	219.1 219.0 218.0 M: 218.7	211.1
Series 2	50 g leaves (15.29 g dry matter), + 3 g $CaCO_3$ aërobic conditions	211.3	211.2	218.3	223.4	223.2	223.2	211.3
Series 3	50 g leaves (15.29 g dry matter), anaërobic conditions	211.3	212.6	216.7	223.5	226.8	231.3	211.0
Series 4	50 g leaves (15.29 g dry matter) + 3 g $CaCO_3$ anaërobic conditions	211.3	214.9	218.9	226.9	234.0	238.8	211.1
Series 5	12 g leaves (3.67 g dry matter) + 25 g soil (21.65 g dry matter)	96.2	96.1	96.2	97.2	97.3	97.2	96.1
Series 6	12 g leaves (3.67 g dry matter) + 25 g soil (21.65 g dry matter) + 2 g $CaCO_3$	96.2	95.9	96.0	97.9	98.5	98.4	96.2

Table 2.

Beech leaves. Mg. nitrogen fixed per 100 g. of the original amount of dry material.

No. of days ⟶	0	36	95	161	229	337
Series 1 Beech leaves under aërobic conditions	0	0	0	31 39 25 M: 32	48 54 49 M: 50	51 50 44 M: 48
Series 2 Beech leaves + $CaCO_3$. Aërobic conditions	0	0	46	79	78	78
Series 3 Beech leaves under anaërobic conditions	0	9	35	80	101	131
Series 4 Beech leaves + $CaCO_3$. Anaërobic condit.	0	24	50	102	148	180
Series 5 Beech leaves + soil	0	0	0	27	30	27
Series 6 Beech leaves + soil + $CaCO_3$	0	0	0	46	63	60

Table 3.

The p_H value of the beech leaves at 6 different times during the duration of the experiment. Where »NO_3« is written under the p_H value, the leaves contained nitrate nitrogen when the p_H measurement was carried out. In all other cases the leaves were free from nitrate.

No. of days ⟶	0	36	92	156	233	337
Series 1 Beech leaves under aërobic conditions	4.8	6.1	6.7	7.0	5.4 NO_3	5.1 NO_3
Series 2 Beech leaves + $CaCO_3$. Aërobic conditions	7.7	7.5	7.5	7.6	7.6	7.7
Series 3 Beech leaves under anaërobic conditions	4.8	5.9	6.5	6.5	6.5	6.5
Series 4 Beech leaves + $CaCO_3$. Anaërobic condit.	7.7	7.6	8.0	8.0	8.2	8.0
Series 5 Beech leaves + soil	5.2	5.5	6.1	6.0	5.3 NO_3	4.9 NO_3
Series 6 Beech leaves + soil + $CaCO_3$	7.8	7.6	8.1	8.2	7.8	8.3

Table 4.

Loss of dry matter (per cent of the original dry matter) undergone by beech leaves at 3 different times during the experiment.

No. of days →	98	200	330
Series 1 Beech leaves under aërobic conditions	15.4	28.0	34.5
Series 2 Beech leaves + $CaCO_3$. Aërobic condition	11.4	22.2	27.0
Series 3 Beech leaves under anaërobic conditions	7.2	12.4	15.3
Series 4 Beech leaves + $CaCO_3$. Anaërobic conditions	9.7	15.3	17.4

Table 5.

Percentage nitrogen in beech leaves (dry matter) at 3 different times during the experiment.

No. of days →	0	98	330
Series 1	1.38	1.64	2.19
Series 2	1.38	1.60	2.01
Series 3	1.38	1.53	1.76
Series 4	1.38	1.58	1.90

corresponds to a 13 % increase in the original amount of nitrogen. It appears from fig. 1 that the increase of nitrogen is greater under anaërobic conditions than under aërobic, and that the addition of calcium carbonate has promoted nitrogen fixation both under aërobic and anaërobic conditions. It further appears that in the two series (3 and 4) where the decomposition took place under anaërobic conditions the fixation does not appear to be finished after 337 days. In the remaining 4 series, however, the increase in nitrogen has ceased after 170—200 days. In the case of series 1 and 5, the point at which nitrogen fixation ceases coincides with the point at which nitrification begins (see table 3),

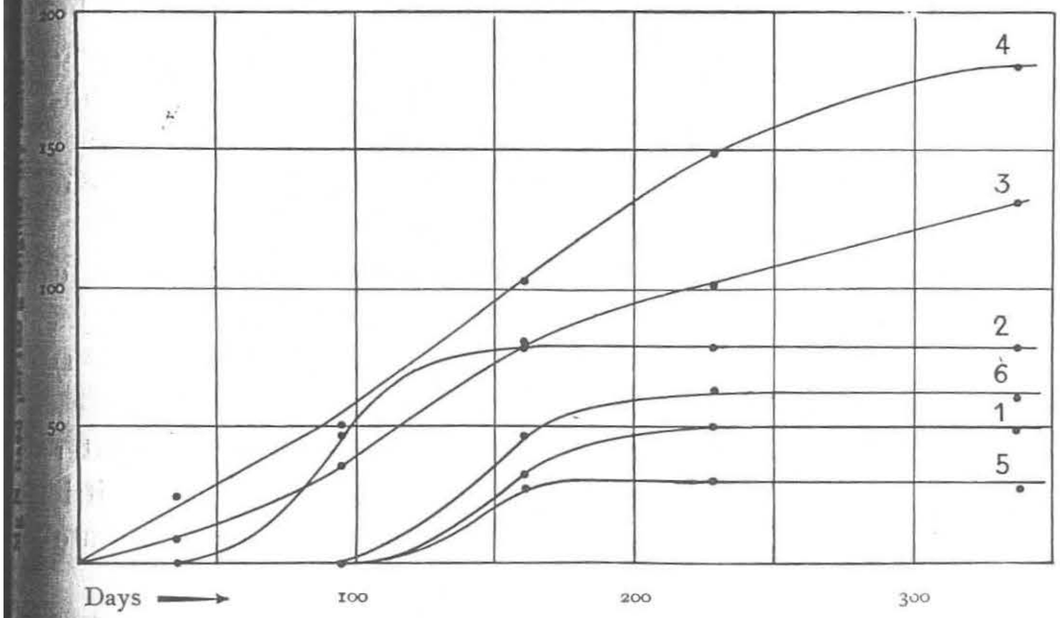

Fig. 1. Beech leaves. Mg nitrogen fixedper 100 g original dry matter. The numbers over the curves show the number of the series.

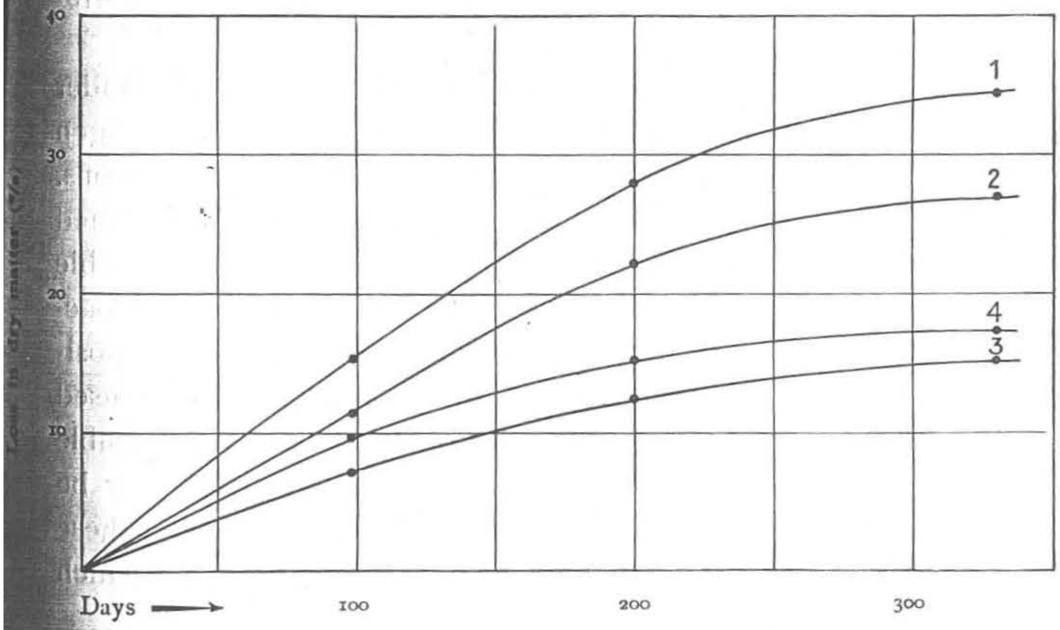

Fig. 2. The loss in dry matter undergone by beech leaves, reckoned as percent of the original dry matter. The numbers over the curves show the numbers of the series.

since as previously mentioned, the presence of nitrate will prevent any further nitrogen fixation. On the other hand, no nitrate or ammonia nitrogen could be detected at any period in series 2 and 6, so that the early cessation of nitrogen fixation in these two series must be due to some other cause.[1]) This may be either that the organic substances necessary for the nitrogen-fixing organisms are no longer formed, because the parent substance (e. g. cellulose) is already completely decomposed, or that the micro-organisms are poisoned by products of metabolism accumulated during the decomposition.

It is seen from table 3 that in series where calcium carbonate has not been added, the p_H value of the beech-leaves increases initially during the decomposition, both under aërobic (series 1) and anaërobic (series 2) conditions.

In series 1 and 5 the p_H value began to decrease at a later period (after about 230 days) because, as mentioned before, nitrification set in. In the remaining series, no ammonia or nitrate was detectable at any period of the investigation.

It is further easily seen by comparing fig. 1 and table 5 that the p_H value of the leaves has an essential effect upon the course of the nitrogen fixation. In all cases where calcium carbonate has been added more nitrogen has been fixed than in the corresponding series without addition.

Fig. 2 shows that the loss of dry matter is much greater under aërobic conditions than under anaërobic.[2]) The greatest nitrogen fixation has however taken place under anaërobic conditions. This can of course mean that the decomposition products formed under anaërobic conditions differ from those formed under aërobic conditions, and are more easily used by the nitrogen-fixing bacteria, or that a larger proportion of the products of decomposition under anaërobic conditions are such that they can be used by the nitrogen-fixing micro-organisms. It is however possible that the slower progress of the decomposition processes may be the reason that larger amounts of nitrogen are fixed under these conditions, since the formation of decomposition products which can serve as a source of carbon for the nitrogen-fixing bacteria presumably lasts much longer under anaërobic than under aërobic conditions.[1] If this explanation is correct, other factors which diminish the velocity of decomposition, e. g. lower temperature, should have a similar effect upon the amount of nitrogen fixed. It is therefore possible that in the forest bed more nitrogen is fixed than in the experiments described above, since the temperature is considerably lower than that at which the beech leaves were kept in the laboratory (22° C). It would therefore be of interest to investigate the effect of temperature upon the process of nitrogen fixation in beech leaves, but such experiments have not yet been carried out.

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[1]) At the conclusion of the experiment some material still available from series 2 and 6 was employed in an analysis for ammonia and nitrate nitrogen according to the method given by Carsten Olsen (1929) (extraction with potassium chloride solution and hydrochloric acid), in which the adsorbed ammonia also appears in the results. No ammonia or nitrate nitrogen was found.

[2]) It also appears from tables 3 and 4 that the velocity of decomposition of beech leaves under aërobic conditions is greater at p_H 6—7 (series 1) than at p_H 7.5—7.7 (series 2). The reverse is the case under anaërobic conditions (series 3 and 4).

Table 5 shows the percentage nitrogen content of the beech leaves at 3 different times during the experiment. It is seen that the nitrogen content increases steadily during the decomposition and this is naturally due partly to the fact that nitrogen is fixed during the process of decomposition, and partly that a loss of nitrogen-free organic matter takes place. The greatest final nitrogen content (2.2 %) was reached in series 1, which is chiefly due to the fact that the loss of dry matter was greater here than in the other series. As previously mentioned, nitrification had set in in the material in series 1, which was not the case in series 2—4. It can probably be concluded from this that beech leaves begin to nitrify when the nitrogen content (calculated on dry matter) has reached a content of about 2.2 %. Old, much decomposed and nitrifying beech leaves in the forest bed contain as a rule about 2 % nitrogen in the dry matter.

c. *Investigation of nitrogen fixation in oak leaves.*

The experiments with oak leaves were carried out in exactly the same way as those with beech leaves. The weighed out portions (each 40 g containing 11.15 g dry matter and 135.3 mg nitrogen) were divided into 4 series, each of which was treated in the same way as the corresponding series with beech leaves. In series 1 and 3 the material had a p_H of 4.5, while in series 2 and 4 2 g calcium carbonate was added to each portion, which altered the p_H value to ca. 7.1. In series 1 and 2 the conditions were aërobic, enough distilled water being added to give a water content of 75—80 %. In series 3 and 4 the conditions were anaërobic, water being added to give the whole the consistency of thin porridge.

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[1] Thus cellulose, which probably decomposes to give products used by nitrogen-fixing organisms, is decomposed much more quickly under aërobic than under anaërobic conditions.

Two other series were also carried out (5 and 6) in which the leaves were mixed with forest soil. Each portion in these two series contained 12 g leaves (3.34 g dry matter containing 40.6 mg nitrogen) and 25 g soil (21.3 g dry matter containing 54.6 mg nitrogen). The mixture of leaves and soil had a p_H value of 4.8 (series 5). In series 6 1 g calcium carbonate was added to each portion, whereby the p_H value was changed to 7.5. The water content of the mixtures was ca. 44 % during the experiments.

The jars containing oak leaves stood together with those containing beech leaves under exactly the same conditions. The experiment was begun 19/12 1930 and finished 10/11 1931 after 327 days. During this period portions were taken to determine the nitrogen content and p_H at 6 different times. At three different times portions were also taken to determine the dry matter. Just as in the case of the beech leaves, 0.5 g sublimate was added to one portion in each series at the commencement of the experiment. These portions were analysed at the conclusion of the experiment (see table 6).

The experimental results are given in tables 6—10 and figs. 3 and 4. It is seen that the greatest increase in nitrogen took place in series 2, where 170 mg nitrogen was fixed per 100 g of the original dry matter, corresponding to a 14.1 % increase in the original amount of nitrogen. The results differ also in several respects from the corresponding ones for beech leaves. Thus the addition of calcium carbonate has in all cases greatly promoted nitrogen fixation (series 2, 4 and 6, fig. 3) while there is little fixation (series 3 and 5) or none at all (series 1) where no calcium carbonate has been added. This is doubtless connected with the lower p_H value of the oak leaves and the fact that the p_H value neither rises so rapidly during decomposition nor reaches such high values as in the case of beech leaves (see table 8)[1]. The p_H value seems in general to be of great impor-

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[1] The reason for this is almost certainly that oak leaves contain large amounts of tannic acids, which are very resistant to decomposition.

Table 6.

The nitrogen content (expressed as mg N) in the weighed out portions of oak leaves at 6 different times during the duration of the experiment

No. of days →		0	41	92	159	221	327	Sublimate added
Series 1.........	40 g leaves (11.15 g dry matter), aërobic conditions	135.2	135.1 135.0 135.3 M: 135.1	135.1 135.2 135.4 M: 135.2	135.4 135.4 135.4 M: 135.4	135.0 135.3 135.2 M: 135.2	135.2 135.3 135.3 M: 135.3	135.0
Series 2.........	40 g leaves (11.15 g dry matter) + 2 g $CaCO_3$ aërobic conditions	135.2	137.2	150.6	153.0	154.3	154.2	135.2
Series 3.........	40 g leaves (11.15 g dry matter), anaërobic conditions	135.2	135.7	136.3	136.4	137.0	138.8	135.3
Series 4.........	40 g leaves (11.15 g dry matter) + 2 g $CaCO_3$ anaërobic conditions	135.2	137.1	143.9	147.9	147.5	147.4	135.1
Series 5.........	12 g leaves (3.34 g dry matter) + 25 g soil (21.3 g dry matter)	95.2	95.2	96.1	96.4	96.4	96.3	95.0
Series 6.........	12 g leaves (3.34 g dry matter) + 25 g soil (21.3 g dry matter) + 1 g $CaCO_3$	95.2	95.0	96.1	98.0	99.3	99.2	95.1

Table 7.

Oak leaves. Mg nitrogen fixed per 100 g of the original amount of dry material.

No. of days →	0	41	92	159	221	327
Series 1 Oak leaves under aërobic conditions	0	0	0	0	0	0
Series 2 Oak leaves + $CaCO_3$. Aërobic conditions	0	18	138	160	171	170
Series 3 Oak leaves under anaërobic conditions	0	4	10	11	16	32
Series 4 Oak leaves + $CaCO_3$. Anaërobic conditions	0	17	78	114	110	109
Series 5 Oak leaves + soil	0	0	28	37	37	34
Series 6 Oak leaves + soil + $CaCO_3$	0	0	28	85	124	121

Table 8.

The p_H value of the oak leaves at 6 different times during the duration of the experiment. Where »NO_3« is written under the p_H value, the leaves contained nitrate nitrogen when the p_H measurement was carried out. In all other cases the leaves were free from nitrate.

No. of days →	0	41	92	159	221	327
Series 1 Oak leaves under aërobic conditions	4.5	4.9	5.2	5.4	5.9	6.6
Series 2 Oak leaves + $CaCO_3$. Aërobic conditions	7.0	7.2	7.2	7.5	7.5	7.5
Series 3 Oak leaves under anaërobic conditions	4.5	4.9	5.2	5.6	5.6	5.6
Series 4 Oak leaves + $CaCO_3$. Anaërobic conditions	7.2	7.5	7.5	7.5	7.6	8.0
Series 5 Oak leaves + soil	4.8	5.0	5.4	5.7 NO_3	5.1 NO_3	4.7 NO_3
Series 6 Oak leaves + soil + $CaCO_3$	7.5	7.5	7.5	7.5	7.5 NO_3	7.4 NO_3

Table 9.

Loss of dry matter (per cent of the original dry matter) undergone by oak leaves at 3 different times during the experiment.

No. of days ⟶	97	200	327
Series 1 Oak leaves under aërobic conditions	18.1	27.2	31.9
Series 2 Oak leaves + $CaCO_3$. Aërobic conditions	19.7	30.5	35.7
Series 3 Oak leaves under anaërobic conditions	9.38	17.5	23.5
Series 4 Oak leaves + $CaCO_3$. Anaërobic conditions	12.6	21.7	27.9

Table 10.

Percentage nitrogen in oak leaves (dry matter) at 3 different times during the experiment.

No. of days ⟶	0	98	330
Series 1	1.21	1.48	1.77
Series 2	1.21	1.65	2.14
Series 3	1.21	1.35	1.64
Series 4	1.21	1.45	1.84

tance for nitrogen fixation in leaves. If p_H is lower than 5.0 the process appears unable to take place. If p_H is between 5 and 5.6 (series 3 and 5, see fig. 3 and table 8) a small amount of nitrogen is fixed. At higher p_H values (7.2—7.5) the nitrogen fixation is much more considerable (2, 4 and 6).

In series 2, 4 and 6, in which calcium carbonate has been added to the leaves, by far the greater part of the nitrogen has been fixed in the first half of the period observed, and has also taken place in a relatively short time, since the curves in fig. 3 rise much more sharply than the corresponding curves (fig. 1) in the case of beech leaves. This is probably connected with the

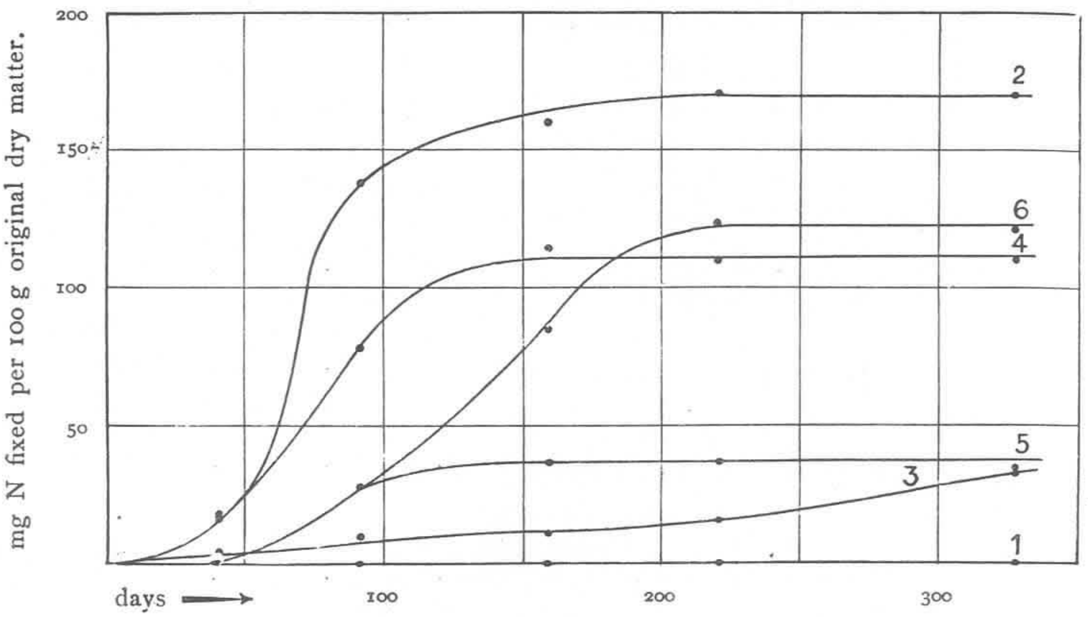

Fig. 3. Oak leaves. Mg nitrogen fixed per 100 g original dry matter. The numbers over the curves show the number of the series.

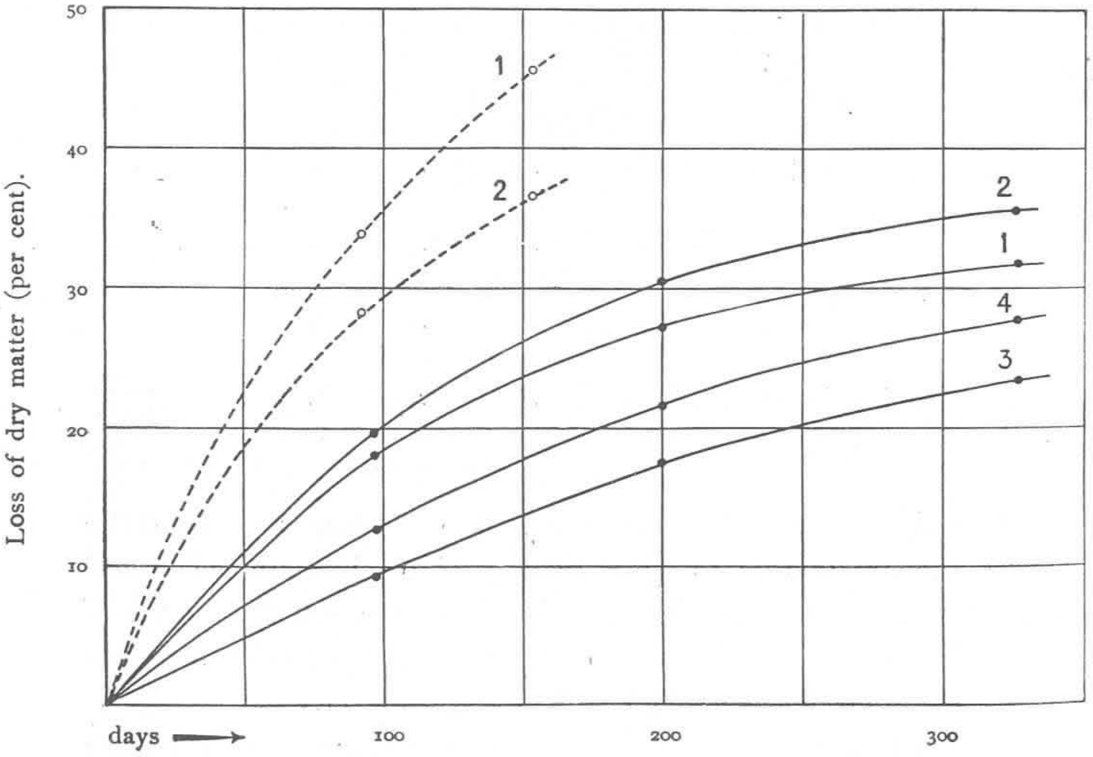

Fig. 4. Loss of dry matter undergone by oak and hornbeam leaves, reckoned as percent of the original dry matter. The continuous curves refer to oak leaves, the dotted curves to hornbeam leaves. The number over the curves refer to the number of the series.

fact that the velocity of decomposition of the oak leaves in the first half of the period observed was considerably greater than for the beech leaves (cf. tables 4 and 9 and figs. 2 and 4), so that there were present in the oak leaves during this period large quantities of decomposition products which could be utilized by the nitrogen fixing micro-organisms. At a later stage nitrogen fixation ceased, presumably because these decomposition products were no longer formed. This supposition is the most probable at least for series 2 and 4, in which no nitrate or ammonia could be detected in the material throughout the experiment. In series 5 and 6, however, the point at which nitrogen fixation ceased coincides with the point at which nitrification set in (table 8), and, as previously stated, the commencement of nitrification will cause nitrogen fixation to cease.

It appears from table 8 that no ammonia or nitrate nitrogen could at any time be detected in series 1. The complete absence of nitrogen fixation in this series is probably due to the low p_H value obtaining in the leaf material at the time when the most vigorous decomposition took place.

Table 10 gives the percentage nitrogen present in the oak leaves at three different points during the experiment. The highest final nitrogen percentage is shown by the material in series 2 (2.14 %), although, as mentioned previously, nitrification had not yet commenced.

d. Investigation of nitrogen fixation in hornbeam leaves.

The weighed out portions (each 40 g, containing 11.88 g dry matter and 207.7 mg nitrogen) were divided into two series. In series 1 the material had a p_H of 4.0, while 2 g calcium carbonate was added to each portion in series 2, which altered p_H to 7.6. The conditions in both series were initially aërobic (water content 76 %) and the jars stood at 22° C. in the same cupboard as the jars with beech leaves and oak leaves.

The experiments were concluded after 154 days. During this time no nitrogen fixation had taken place, and it is not to be anticipated that any would take place later, since ammonia formation (but not nitrification) rapidly set in in the material. It is seen from table 11 that the nitrogen content of the weighed out portions of hornbeam leaves remained practically speaking constant

Table 11.

Nitrogen content (in mg N) in the weighed out portions of hornbeam leaves at 4 different times during the experiment. The p_H values of the leaves at the same times are also given.

No. of days ⟶	0	36	87	154
Series 1 40 g leaves (11.88 g dry matter)	207.7 p_H=4.0	208.0 p_H=5.4	208.1 p_H=6.2	207.0 p_H=6.9
Series 2 40 g leaves (11.88 g dry matter) + 2 g $CaCO_3$	207.7 p_H=7.6	208.3 p_H=7.5	208.2 p_H=7.5	208.2 p_H=7.6

Table 12.

Loss of dry matter (per cent of the original dry matter) undergone by hornbeam leaves at 2 different times during the experiment.

No. of days ⟶	92	153
Series 1	33.9	45.6
Series 2	28.1	36.3

throughout the whole experiment. In the case of series 1, it is seen from table 11 that the p_H value increased very considerably (from 4.0—6.9) in the course of the experiment. It appears from table 12 that a very vigorous decomposition of the leaves took place simultaneously, since nearly half of the dry matter of the leaves (in series 1) had disappeared after 154 days (see also fig. 4). As a consequence of this very vigorous decomposition the water content of the leaves increased considerably, the loss in dry matter being greater than the loss by evaporation. In this way the conditions became partly anaërobic, which is certainly the reason that only ammonia formation and not nitrification set in.

The rapid decomposition of hornbeam leaves is due among other things to their comparatively high nitrogen content. The leaves contained initially 1.75 % nitrogen in the dry material and at the conclusion of the experiment the nitrogen percentage had risen to ca. 3.2 %. The early formation of ammonia, due to the larger nitrogen content in conjunction with the rapid decomposition, is certainly the reason that no nitrogen fixation took place in the material.

It is remarkable that it was hornbeam leaves in which Henry found a specially large increase in nitrogen. Apart from the fact that his figures for the increase in nitrogen are certainly much too high, on account of his defective technique, this may be because the hornbeam leaves in Henry's experiments contained initially only 0.947 % nitrogen in the dry material, while the nitrogen percentage in the hornbeam leaves used in the present work was nearly twice as high. The hornbeam leaves used by Hornberger contained 1.36 % nitrogen, and withered leaves just fallen from a hornbeam at Carlsberg in the autumn contained 2.3 % nitrogen. It seems thus that the nitrogen content of fallen hornbeam leaves can vary within wide limits.

e. Discussion of the collected experimental results.

It appears from the experiments described above that a considerable nitrogen fixation can take place in fallen beech and oak leaves. In the case of beech leaves under the most favourable conditions 180 mg nitrogen was fixed per 100 g of the original dry matter, which corresponds to a 13 % increase in the original amount of nitrogen. In the case of oak leaves, the largest amount of nitrogen fixed was 170 mg per 100 g of the original dry matter, which corresponds to a 13 % increase in the original amount of nitrogen. These results have however only been obtained in series where the leaves have been mixed with calcium carbonate. In leaves not mixed with calcium carbonate the amount of nitrogen fixed in the most favourable case was 131 mg nitrogen per 100 g original dry matter (beech leaves series 3), corresponding to a 9.5 % increase in the original amount of nitrogen.

The experiments carried out thus confirm Henry's assertion that fixation of free nitrogen can take place in fallen leaves, although the increases in nitrogen found in the Carlsberg laboratory are not as large as those mentioned by Henry in his first paper.

It is clear from the collected results that the p_H values of the leaves is of extremely great importance for the progress of nitrogen fixation. No fixation at all takes place at p_H values lower than 5.0, a comparatively negligible fixation with p_H between 5.0 and 6.0, while p_H values between 6.0 and 7.7 are the most favourable for the process.

The question of how far nitrogen fixation takes place in the dead leaves of the forest bed under natural conditions is thus partly a question of what p_H values obtain in the leaves. The p_H value of the leaves is however found to vary considerably with their age and degree of decomposition, and is also more or less dependent on the p_H value of the underlying soil. The last factor is specially important in even and flat land, where the leaf layer is thin. Some examples of this are shown in table 13[1]). The experiments were carried out in November and the table shows the p_H value of old decomposing beech leaves, while the p_H value of the top layer of leaves which had fallen the same autumn was not measured. It is seen that the p_H value of the leaves decreases with decreasing p_H values in the underlying soil, but that the leaves on an acid soil have always a higher p_H value than the soil. The matter is somewhat different in ditches and in hollows in uneven country, where the layer of leaves is thick. Table 14 shows the p_H value at different depths under these conditions. It is seen that the p_H value of the freshly fallen beech leaves lies between 4.9 and 5.4, and is thus fairly independent of the p_H value of the soil. At a depth of 10—20 cm p_H is considerably higher, and even above raw humus p_H can be about 6.0 at this depth. At greater depths p_H decreases again on a strongly acid soil, since the p_H value of the underlying soil begins to have an effect.

It may be concluded from these investigations that the conditions for nitrogen fixation in fallen leaves are specially favourable in forests with neutral or alkaline soil, but very unfavourable in forests with a strongly acid soil. It is however possible that a not inconsiderable nitrogen fixation can take place in hollows where the layer of leaves is thick, even in woods with a strongly acid soil.

The above investigations of nitrogen fixation in beech and oak leaves do not permit of any conclusions as to how much

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[1]) A considerably greater number of investigations have been carried out, but in order to save space only a few typical examples have been given.

Table 13.

p_H values of decomposing beech leaves and underlying soil. The measurements were carried out in November, 1931.

	Decomp. leaves	Underlying soil
Raw humus in Grib Skov	4.8	3.8
Mould in Grib Skov	6.0	5.7
Mould in Ermelunden	6.2	5.4
Mould in Store Bøgeskov	7.0	7.0
Mould in Allindelille Fredskov	7.5	7.7

Table 14.

Beech leaves. p_H values at various depths in places where the layer is fairly thick (hollows in uneven ground and ditches). The measurements were carried out in November 1931.

	Raw humus Grib Skov	Raw humus Ermelunden	Ermelunden	Ermelunden. Ditch	Valborup Skov	Valborup Skov. Ditch	Allindelille Fredskov	Store Bøgeskov
0—3 cm's deep	4.9	5.2	5.4	5.3	5.2	5.0	5.4	5.3
ca. 10 cm's deep	6.0	6.1	7.0	6.6	6.5	7.3	7.5	7.5
ca. 20 cm's deep	5.8	5.9	7.0	7.0	6.5	7.3	—	7.5
ca. 30 cm's deep	4.8	5.4	—	7.0	—	—	—	—
Underlying soil	3.7	4.1	7.0	7.5	6.6	7.5	7.7	7.2

free nitrogen can be fixed in forest leaves under natural conditions, since it must be remembered that the experimental conditions were different from natural conditions in the forest bed. Among other things, the experiments were carried out at a temperature (22°) considerably higher than that in the forest bed.

It is however by no means certain that less nitrogen will be fixed at a lower temperature (e. g., 15 ° C) than in the experiments described above. At a lower temperature the leaves will be decomposed more slowly, and, as previously mentioned (p. 21) a not too rapid decomposition seems to favour the fixation process. Investigations of the effect of temperature on nitrogen fixation in forest leaves would therefore be of great interest.

Another factor which may be of importance for nitrogen fixation in leaves under natural conditions is the rain which in soaking through may remove substances or products of metabolism which are harmful for the fixation process. It is possible that such products accumulate in the leaves in laboratory experiments, and may be a contributory cause to the cessation of fixation after a certain time. The tannic acids in oak-leaves, which undoubtedly prevented any fixation in series 1 (tables 6 and 7) would also presumably be washed away under natural conditions. It is however possible to arrange laboratory experiments so that the leaves can be washed through with distilled water at suitable intervals. Such experiments have already been started (at a temperature below 22 ° C) and the results will be published when the experiments are finished.

No investigations have been carried out as to which microorganisms are responsible for nitrogen in beech leaves and oak leaves. At the close of each experiment, however, a portion of the leaf material was in all cases transferred to a culture containing mannite.[1]) Since in no case did any Azotobacter skin appear on the surface of the liquid after standing, it can be stated with certainty that the nitrogen fixation in the leaves is not due to the action of Azotobacter. There developed however, small stick-shaped bacteria in the mannite solution, as well as Clostridium forms, and a smell of butyric acid appeared. After standing for 3 weeks at 30°, 2.0—3.5 mg nitrogen was fixed per 1 g of mannite added. Clostridium pastorianum under similar conditions fixes comparable amounts of nitrogen.

It is doubtful whether the large quantities of nitrogen compounds found in the beds of forests are due to nitrogen fixation in fallen leaves, and this is specially true for strongly acid soils, in which large amounts of ammonia and nitric acid are also formed each year. It is true that the amount of nitrogen removed in timber after the felling of trees is only small, and is only removed at intervals of many years, but the rain in soaking through carries considerable amounts of nitrogen (especially as nitrate) to the lower soil layers. It is therefore probable that a fixation of nitrogen also takes place in forests with a strongly acid soil. The experiments described here show that this fixation can hardly take place in the leaves by the action of free living micro-organisms. It is reasonable to suppose that the Mycorrhiza fungi (which occur specially with acid soils) are able to fix nitrogen, though all attempts to show this have hitherto given negative results. This may however only be because the correct experimental conditions have not been present in the cultures used.

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[1]) This contained per litre: 20 g mannite, 1 g secondary potassium phosphate, 0.2 g magnesium sulphate, 10 mg manganous sulphate and 20 mg ferric citrate. 50 cc of this solution plus 0.5 g calcium carbonate were used in each experiment.

SUMMARY.

Fixation of free atmospheric nitrogen can take place in beech and oak leaves during the process of decomposition.

In the experiment with beech leaves 180 mg nitrogen per 100 g original dry matter (corresponding to an increase of 13 % in the original amount of nitrogen) was fixed in the most favourable case.

In the experiment with oak leaves the largest amount of nitrogen fixed was 170 mg per 100 g original dry matter, corresponding to a 14 % increase in the original amount of nitrogen.

These results were however only obtained in cases in which the leaves had been mixed with calcium carbonate. In leaves which had not been mixed with calcium carbonate, the amount of nitrogen fixed in the most favourable case (with beech leaves) was 131 mg nitrogen per 100 g original dry matter, corresponding to a 9.5 % increase in the original amount of nitrogen.

The p_H value of the leaf material is of great importance for the fixation of nitrogen. No fixation takes place at p_H values lover than 5.0. A comparatively insignificant fixation can take place at p_H 5.0—6.0, while values of p_H between 6.0 and 7.7 are favourable to the process.

Beech and oak leaves remain free from any trace of ammonia or nitrate nitrogen for long periods (up to one year) during their

decomposition, the nitrogen compounds being retained by the micro-organisms. The nitrogen percentage in the dry matter increases steadily during this period, partly because free nitrogen is fixed, and partly because a loss of dry matter takes place. When the nitrogen percentage has reached ca. 2.2 %, nitrification sets in, and nitrogen fixation no longer takes place. In many cases, however, fixation of free nitrogen ceases at a much earlier point, probably because the organic decomposition products necessary for the nitrogen fixing micro-organisms are no longer formed.

In the experiments carried out with hornbeam leaves, no fixation took place. This is due to the high nitrogen content of hornbeam leaves (1.75 % nitrogen in the dry material) and the consequent rapid decomposition, which caused ammonia formation to set in at an early period.

The nitrogen fixation in beech and oak leaves is not due to the action of Azotobacter, since this bacteria was not present in the leaves. Clostridium forms were however found to be present.

The author wishes to tender thanks to Professor S. P. L. Sørensen, chief of the Carlsberg Laboratory, for the never-failing interest with which he has followed this work.

August 1932.

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